COMPARATIVE *IN VITRO* PULMONARY TOXICITY OF ENGINEERED, MANUFACTURED, AND ENVIRONMENTAL NANOPARTICLES

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ABSTRACT

Engineered nanoparticles display many novel physicochemical properties for a variety of applications. Due to these novel properties and applications nanoparticles may have unique routes of exposure and toxicity. This study examines the: 1) ability of the MTT and WST-1 assays to generate false positives or negatives when examining the cellular toxicity of single wall carbon nanotubes (SWCNTs); 2) comparative *in vitro* pulmonary toxicity of 4 different, >90% purified, SWCNTs (#1-4), nanographite fiber (NGF), ultrafine carbon black particles (UFCB), diesel exhaust particles (J-DEP), coal fly ash (CFA) and residual oil fly ash particles (ROFA); and 3) relationship between nanoparticle surface reactivity and *in vitro* pulmonary toxicity. MTT and WST-1 assays did not produce either false positive or negative results since SWCNTs alone were negative in each assay. BEAS-2B cells, a human bronchiolar epithelial cell line, were exposed to various concentrations of each type of nanoparticle as well as several combustion particles and cellular effects determined using the MTT, WST-1, LDH, and direct cell counting assays at 24 hr post-exposure. SWCNT induced cellular effects and trends in toxicity were found to be dependent upon the toxicity test assay employed. SWCNTs displayed differential cellular toxicity amongst themselves as well as comparable toxicity to certain combustion particles. *In vitro* wound-repair and gene expression profiling studies confirmed differential toxicity of purified SWCNTs. SWCNTs inhibited cell growth whereas combustion particles induced cellular cytotoxicity in BEAS-2B cells. Nanoparticles and combustion particles were tested for their ability to generate thiobarbituric acid reactive substances (TBARS) in an acellular assay. TBARS analysis demonstrated the following hierarchy of reactivity: ROFA >> J-DEP > CFA = NGF = UFCB = SWCNTs #1- #4 and demonstrated that, in contrast to certain types of combustion particles, SWCNTs cellular effects were not due to their surface reactivity. These results demonstrate the need for *in vitro* test methods that can accurately determine SWCNT pulmonary toxicity and ultimately predict their *in vivo* toxicity. (Funding: USEPA-NCSU Cooperative Training Agreement CT 829470. This abstract does not necessary reflect EPA Policy)

OBJECTIVES

Research was conducted in order to:

1. compare the relative *in vitro* pulmonary toxicities of a variety of purified (>90%) SWCNTs obtained from different primary commercial suppliers;
2. compare various *in vitro* test methods to determine the cellular effects of SWCNTs on human airway cells;
3. compare the relative *in vitro* pulmonary toxicities of a variety of purified (>90%) SWCNTs with other manufactured nanoparticles and fine (<2.5µm) particles from primary combustion sources which contribute to ambient air particulate pollution;
4. determine the role of surface reactivity in SWCNT effects on human airway cells.

MATERIALS AND MEHTODS

Engineered, Manufactured Nanoparticles and Environmental Particles: SWCNTs were obtained from 4 different primary commercial sources and designated SWCNT-1 (ave. dia. 1.3nm, surface area 300-600 m²/g), -2 (ave. dia. 1nm), -3 (ave. dia. 1.4nm), and -4 (ave. dia. 1.2nm). All SWCNTs were >90% as specified by each primary supplier. Printex 90 carbon black (UFCB) (dia. 14nm, surface area 300 m²/g) was obtained from Degussa. Nanographite fiber (NGF) (ave. dia. 100-200nm) was obtained from Sigma Chemical Co. St. Louis, MO. Coal fly ash (CFA) containing fine (<2.5µm) particles was derived from the combustion of Western Kentucky bituminous coal and obtained from the National Risk Management Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC (Gilmour et al., *J. Air & Waste Mange. Assoc.* 54:286-295, 2004). Japanese diesel exhaust particles (J-DEP), containing fine/ultrafine particles <200nm, was derived from a Toyota diesel truck engine and provided by Dr. Kobayashi, National Institute for Environmental Studies, Tsukuba, Japan (Kobayashi and Ito, *Fundam. Appl. Toxicol.* 27:195-202, 1995). Residual oil fly ash (ROFA) containing fine (<2.5µm) particles was obtained from a utility power plant burning low sulfur #6 residual oil and has been extensively characterized (Dreher et al., *J. Toxicol. Environm. Health* 50:285-305, 1997).

Particle Reactivity: All particles were examined for their ability to generate thiobarbituric acid reactive substances (TBARS) as described by Pritchard et al., *Inhal. Tox.* 8:457-477, 1996, and Molinelli et al., *Inhal. Tox.* 14:1069-1086, 2002.

In Vitro Pulmonary Cell Model: The BEAS-2B human bronchial cell line (S6 subclone, passages 68-91) was employed in these studies and represents an immortalized line of normal human bronchial epithelium derived by transfection of primary cells with simian virus-40 early region genes. BEAS-2B were grown/maintained in culture as described by Molinelli et al., *Inhal. Tox.* 14:1069-1086, 2002.

Nanoparticle and Ultrafine/Fine Combustion Particle In Vitro Exposures: Stock suspensions of particles were prepared in KGM media containing fetal bovine serum (FBS) and sonicated using a probe set at 6-8W for 2 min. Dosing suspensions of particles were prepared by diluting stock particle suspensions in KGM/FBS followed by additional probe sonication set at 6-8W for 2 min. All particle dosing suspensions were kept in a ultrasonic water bath until used to expose BEAS-2B cells. Final concentration of FBS was <0.2%. BEAS-2B cultures were exposed to various concentrations of particles and toxicity assessed by 4 different cellular assays 24h post-exposure.

Cellular Toxicity Assays: BEAS-2B cellular toxicity was assessed by: 1) MTT Cell Proliferation Assay (ATCC, Manassas, VA); 2) Cell Proliferation Reagent WST-1 (Roche Diagnostics, Mannheim, Germany); 3) Lactate dehydrogenase release was assessed using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI); 4) cell number - BEAS-2B cells were trypsinized and cell number/viability as well as cells containing SWCNTs was determined by visual counting of cells using a Nikon microscope and hemocytometer following addition of trypan blue (Gibco, Grand Island, NY). For MTT, WST-1, and LDH commercial assays, only minor modifications to the manufacturer's recommended protocol were employed in order to minimize interference by particles in each assay.

In Vitro Wound Repair Assay: BEAS-2B cells were grown to confluence. A consistent size wound was produced in each culture by scraping each using the wide bore end of a 1 ml sterile plastic pipet. Cultures were then exposed to various concentrations of purified SWCNTs. Cultures were photographed when control/unexposed wounded cultures had completely repopulated the scrapped or "wounded" area.

RNA Extraction, Characterization, and Quantification: BEAS-2B cells were exposed to KGM/FBS (FBS <0.2% final conc.), SWCNT-2, SWCNT-3, JDEP, or UFCB at a concentrations of 5 µg/ml for 3h. RNA was isolated from cultures using TRIzol as described by the manufacturer (Invitrogen). Isolated RNA was dissolved in 10 mM Tris-HCl, pH 7.0, containing 0.1 mM EDTA and RNAsin Plus RNase inhibitor (Promega, Madison, WI) at 1U/ml. RNA quality was assessed using the Agilent 2100 Bioanalyzer and RNA Nano LabChip™ (Agilent Technologies, Palo Alto, CA). RNA was quantified using the RibroGreen procedure (Molecular Probes, Inc., Eugene, OR). RNA samples were stored at -80°C until analyzed for gene expression profiling.

Gene Expression Profiling and Bioinformatic Analysis: Gene expression analysis of isolated RNA samples was performed by Expression Analysis Inc., Durham, NC, using the Affymetrix platform and Human Genome U133 Plus 2.0 Array Chips (Affymetrix Inc., Santa Clara, CA). Bioinformatic analysis of Affymetrix gene expression data was conducted using GeneSpring version 7.2 software (Silicon Genetics, Redwood City, CA).

PRE-TESTING OF CELLULAR TOXICITY ASSAYS

The following pre-test studies were conducted for each commercial cellular toxicity assay in order to maximum assay sensitivity and minimize the potential for each assay to yield false positive or negative results:

1. cells were plated at densities that were well within the linear range of each commercial assay (1.5 x 10⁴ cells/well of a 96-well plate (~47,000 cells/cm²);
2. for the MTT and WST-1 assays, a washing step was introduced to minimize interference from particles;
3. each assay was run with just particles across the concentrations employed in order to ensure particles did not by themselves react with dyes employed in each assay;
4. for the LDH assay, studies were performed to ensure LDH did not bind to SWCNTs.

In Vitro Pulmonary Cellular Toxicity of Engineered Nanoparticles and Environmental Ultrafine/Fine Particles

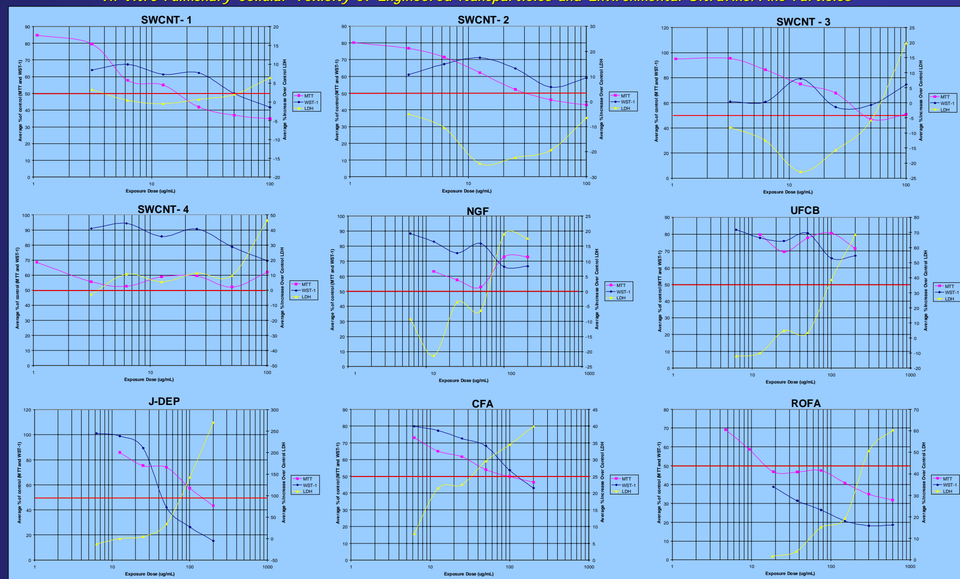

Figure 1. *In Vitro* Pulmonary Cellular Toxicity of Engineered/Manufactured Nanoparticles and Environmental Ultrafine/Fine Particles.
BEAS-2B cells were exposed to various concentrations of: 4 purified (>90%) SWCNTs (#1-#4) obtained from different primary suppliers; 2 types of manufactured nanoparticles, UFCB, and NGF; as well as to three different combustion source particles, J-DEP, CFA, and ROFA in order to compare their relative *in vitro* pulmonary toxicities. Cellular effects were determined at 24h post-exposure by a variety of commercially available toxicity test assays (MTT, N=22; WST-1, N=8; LDH, N=8). MTT and WST-1 assays gave very different dose-response curves for SWCNT-1 through SWCNT-4, NGF, and UFCB. In contrast, the MTT and WST-1 assays tended to provide "more consistent" dose-response curves for combustion derived ultrafine/fine particles. Decreases in cell number for SWCNT-1, 2, 3, and 4 were not associated with corresponding significant increases in LDH indicating inhibition of cell growth. In contrast, decreases in BEAS-2B number following exposure to fine combustion source particles (CFA, J-DEP, ROFA) were associated with increased LDH release indicating cellular cytotoxicity. Interestingly, MTT dose-response curves for SWCNT-1, -2, and -3 were found to alter BEAS-2B cell growth at lower concentrations when compared to fine combustion sources particles with a high elemental carbon content (J-DEP, CFA). Values represent average % change from control (untreated) cultures run in parallel. Standard error (SE) bars were omitted for presentation purposes, SE ranges for each assay were: 3-15% for MTT; 2-16% for WST-1, and 5-13% for LDH, of indicated values.
EC_{50} values for each assay and type of particle that provided a an adequate dose-response curve is depicted in Table 1.

Effect of SWCNTs on BEAS-2B Cell Growth and Viability

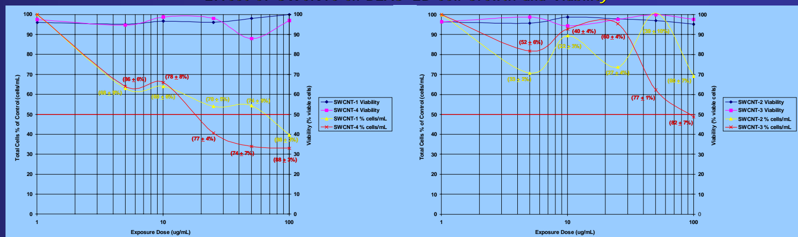

Figure 2. Effect of SWCNTs on BEAS-2B Cell Growth and Viability.
BEAS-2B cells were exposed to various concentrations of four purified (>90%) SWCNTs (SWCNT-1, -2, -3 and -4) obtained from different primary suppliers (N=4 per concentration and SWCNT source). Cell cultures were trypsinized 24h post-exposure and cell number and viability as well as % of cells containing SWCNTs was determined as described in Materials and Methods. Consistent with LDH results in Figure 1, SWCNTs had no effect of BEAS-2B cell viability. However, differential effects of SWCNTs on BEAS-2B cell growth was observed with the following hierarchy: SWCNT-4 > SWCNT-1 > SWCNT-3 while SWCNT-2 had no consistent effect on BEAS-2B cell growth. Values represent average % change from control (untreated) cultures run in parallel. Standard error (SE) bars were omitted for presentation purposes, SE ranges for each assay were: 3-22% for Total Cells and 1.3-10% for Viability, of indicated values. Values in parenthesis indicate the average % of cells containing SWCNTs ± standard deviation.
EC50 values for each assay and type of particle that provided a an adequate dose-response curve is depicted in Table 1.

Table 1
Comparative In Vitro PulmonaryToxicities of Engineered Nanoparticles and Environmental Fine/Ultrafine Particles

Particle	EC^{1}_{50} (µg/ml)		
	Cell Number	WST-1	MTT
SWCNT – 1	65	50	17
SWCNT – 2	>100	>100[2]	30
SWCNT – 3	95	>100	42
SWCNT – 4	18	>100	>100
UFCB	Not Determined	>200	>200
NGF	Not Determined	>200	>200
JDEP	Not Determined	45	150
CFA	Not Determined	120	100
ROFA	Not Determined	~8[3]	15

1. EC_{50} values were derived from data for each assay presented in Figures 1 and 2.
2. For some particles a dose-response relationship could not be obtained and a symbol (>) in front of the highest concentration was used to indicate the EC_{50} resided above this value.
3. ROFA EC_{50} value was extrapolated from Figure 1.

EC_{50} based hierarchy of particle *in vitro* pulmonary toxicity was found to be assay dependent with the following results:

Direct cell counting assay: SWCNT-4 > SWCNT-1 > SWCNT-3 > SWCNT-2
MTT assay: ROFA = SWCNT-1 > SWCNT-2 > SWCNT-3 >> CFA > JDEP > UFCB, NGF, SWCNT-4
WST-1 assay: ROFA > SWCNT-1 = JDEP > CFA > SWCNT-2, 3, 4, UFCB, NGF

NOTE: MTT and WST-1 rankings were different than ranking based on direct cell counting with respects to SWCNTs.

Acellular Assessment of Particle Reactivity

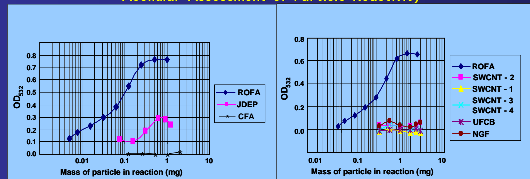

Figure 3. Nanoparticle and Combustion Particle Acellular Reactivity.
All particles were examined for their ability to generate thiobarbituric acid reactive substances (TBARS). Redox active metals are readily detectable in the TBARS assay. ROFA and JDEP were the most reactive particles while purified (>90%) SWCNTs and all other particles were unreactive in the TBARS assay. TBARS analysis demonstrated the following hierarchy of particle reactivity: ROFA > JDEP > CFA = SWCNTs-1 to SWCNT-4 = UFCB = NGF. These results suggest that SWCNT inhibition of BEAS-2B cells is not due to reactive metals or direct oxidative stress.

Differential In Vitro Wound Repair of SWCNTs

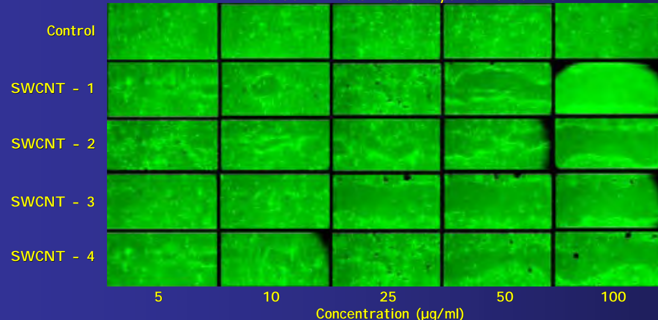

Figure 4. Effect of SWCNTs on *In Vitro* Human Bronchiolar Wound Repair.
BEAS-2B cells were grown to confluence. Afterwards, a consistent size wound was produced in each culture. Wounded cultures were then continuously exposed to various concentrations and types of purified (>90%) SWCNTs. Cultures were photographed when control/unexposed wounded cultures had completely repopulated the scrapped or "wounded" area. Results demonstrate differential capability of each SWCNT to inhibit *in vitro* wound repair. Results demonstrate the following hierarchy in SWCNT inhibition of *in vitro* human airway wound repair: SWCNT-1 = SWCNT-4 > SWCNT-3 > SWCNT-2.

Differential Gene Expression Profile Induction by Engineered Nanoparticles

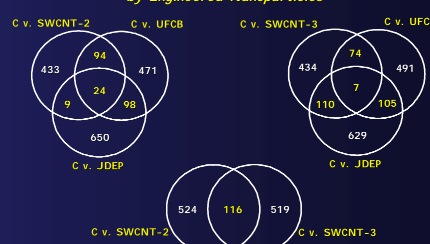

Figure 5. Differential *In Vitro* Pulmonary Gene Expression Profiles Induced By Engineered Nanoparticles and Combustion Ultrafine Particles.
BEAS-2B cells were exposed to either purified (>90%) SWCNT-2 (N=3), SWCNT-3 (N=3), JDEP (N=3), or UFCB (N=3) for 3h as described in Materials and Methods. RNA was isolated from unexposed control (C) and exposed BEAS-2B cells and examined for alterations in gene expression as described in Materials and Methods. Gene lists having statistically significant (p > 0.05) > or < 1.5-fold changes in expression over control (C) cultures were obtained for each particle and subsequently used to compare to each other. Venn diagrams demonstrate that: 1) SWCNTs induce a gene profile which is not very similar to UFCB or JDEP; and 2) SWCNT-2 and SWCNT-3 do not express a very large number of genes in common.

SUMMARY

The overall results of these studies demonstrate:

1. MTT and WST-1 assays provided different assessments for the *in vitro* pulmonary toxicity of purified SWCNTs when compared to a direct cell counting method;
2. there is a differential *in vitro* pulmonary toxicity amongst purified SWCNTs and combustion particles which is assay dependent;
3. purified SWCNTs inhibit BEAS-2B cell growth by a different mode of action and mechanism when compared to ultrafine/fine combustion particles;
4. purified SWCNTs elicit comparable *in vitro* pulmonary toxicity when compared to certain ultrafine/fine combustion particles which have been previously shown to represent a significant public health risk.

Studies are underway in order to:

1. identify the physicochemical properties and mechanisms responsible for the differential *in vitro* pulmonary toxicity of purified SWNCTs;
2. identify more accurate methods to assess the *in vitro* pulmonary toxicity and cellular uptake of purified SWCNTs;
3. determine if the differential *in vitro* pulmonary toxicity of purified SWCNTs can be extrapolated *in vivo*.